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Short report

Gender-related difference in bloodstain RNA ratio stored under uncontrolled room conditions for 28 days

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ABSTRACT

Bloodstain age is a parameter that can be used in crime scene investigations. Bloodstain age can be determined by measuring the 18S rRNA:β-actin mRNA ratio by Reverse Transcription-quantitative PCR (RT-qPCR). Since this ratio is a function of time, it can be used as an estimator of bloodstain age. However, it is important to validate the technique in a variety of scenarios before it can be applied. We investigated 18S rRNA:β-actin mRNA ratio in bloodstains from sixteen Chinese subjects in 28 days under uncontrolled room conditions. The ratio changed in a linear fashion. It was also found that the subjects' gender affected the relationship between time and the RNA ratio.

1. Introduction

1.1. Bloodstains as evidence

Bloodstains are frequently used as evidence when investigating different types of crimes. The presence of bloodstains may be evidence of murder or injury, so the examination of stains found at the crime scene is vital. Evaluation of the distribution and appearance of the bloodstains at the scene is usually the province of specially trained and experienced forensic scientists (e.g., bloodstain pattern analysts). From bloodstains many clues can be drawn about the case. The bloodstain pattern can help to reconstruct the crime scene event; e.g., blood flow patterns from an injury can prove that a body has been moved; DNA can be analyzed from bloodstains to identify a possible suspect. Recent progress in RNA techniques may be used to deduce the possible tissue origin[1–6] and even to determine the bloodstain age.[7,8] This is important because bloodstain age can be very useful in criminal investigations to identify when the crime occurred or to include or exclude an individual as a suspect.

1.2. Bloodstain aging

As bloodstains dry and age, they rapidly lose their initial bright-red color within hours and become a dull red; within days they become brownish. Research on bloodstain aging has included chemical assays,[9] enzyme assays,[10] and many methods utilizing deteriorative changes in the visible spectrum of hemoglobin (Hb) over time.[11–14] Several new approaches have recently been introduced: High Performance Liquid Chromatography (HPLC),[15,16] Atomic Force Microscopy (AFM),[17] Electron paramagnetic Resonance Spectroscopy (EPR),[18,19] Diffuse Reflectance Spectroscopy (DRS)[20] and others. All of these methods have limited effectiveness with either weak age correlation or specific sample type or size constraints, and may be adversely influenced by environmental factors.

With the development of gene expression assays, specific mRNA degradation patterns have been proposed as a new tool for forensic investigations. It is now possible to isolate RNA of sufficient quality and quantity from dried blood, semen, and saliva stains to detect particular mRNA species using RT-PCR methods and to determine their tissue origin.[1–6] RNA techniques have considerable sensitivity, and some researchers have succeeded in identifying some mRNA markers in quite old samples of blood (13–16 years) and saliva (2–6 years).[21] Recently the collaborative exercise on RNA/DNA co-

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analysis for body fluid identification and Short Tandem Repeat (STR) profiling organized by the European DNA Profiling Group (EDNAP) supported the potential use of an mRNA-based system for the identification of blood in forensic casework that is compatible with current DNA analysis methodology.[22,23]

Anderson et al. demonstrated that the ratio between different types of RNA (18S rRNA:β-actin mRNA ratio) changed over time in a linear fashion in bloodstains under controlled conditions.[7] This method was sensitive enough to be used on stains containing as little as 1 μl blood. It was also shown that multivariate analysis of different RNA species could be used to differentiate between samples of different ages in the defined population.[8]

1.3. Confounding factors affecting RNA bloodstain aging methods

Based on the belief that mRNA is more vulnerable than rRNA, the RNA technique to deduce bloodstain age is very promising, but before it can be introduced into practice, it needs to be validated across a varied range of conditions that may apply in practice. Thus, we should find out how the technique is affected by different environmental factors (indoors/outdoors; microorganisms, temperature, humidity, and full-spectrum light, etc.). It is also important to evaluate the effects of age, gender, and ethnicity on RNA bloodstain aging. The same technique might be applied to other biological samples such as saliva and hair. Setzer et al. have shown that it is better to store bloodstains dry at room temperature in plastic bags: housekeeping and tissue-specific mRNA recoverability from blood was shown up to 547 days. When samples were outside with no rain, mRNA recoverability from blood could be shown up to 30 days. Rain had detrimental effects on the recoverability of blood, with mRNA recoverable up to 3 days.[3] Preece et al. showed that gender, age at death, and brain pH, all of them have significant effects upon mRNA levels in the post-mortem human brain.[24] When Anderson et al. examined the relative quantity of β-actin and 18S as a function of time using RT-qPCR, it was suggested that the minimum age difference that could be identified was 4 weeks. Therefore, the present study was designed to be carried under room conditions with no strict control of temperature or humidity, and with daylight and shorter time intervals (0, 7, 14, 21, and 28 days), which aimed to observe the progressive degradation of the two species of RNA.

2. Material and methods

2.1. Blood collection and sampling

Blood was drawn from 16 healthy individuals (eight males and eight females) from the Chinese Han population. Written informed consent was taken from all donors. All of the donors were aged between 20 and 30 years old. 5 ml of blood was collected by venipuncture on three separate occasions from each donor. Ten microlitres aliquots were immediately spotted onto one piece of 100% cotton fabric and dried at room temperature. A separate piece of fabric was used for each individual, but all pieces were taken from one large piece of cloth. The blood collection procedure was performed over a 1-week period. The cloth which was used to collect bloodstains was suspended on a frame near the ceiling of a room maintained at a temperature of 18–22 °C, with relative humidity maintained around 50%. The cloth was not subjected to direct sunlight, but was subject to normal room light. Since bloodstains are often retrieved from rooms in which ambient temperature and humidity vary, it was considered to be a reasonable simulation of conditions that might affect "real" forensic blood samples. Samples were processed when they reached the desired ages of 0 (4 h after blood draw), 7, 14, 21, and 28 days.

2.2. RNA extraction

TRIzol Reagent (Invitrogen, Carlsbad, CA) was used to isolate RNA from the dried blood samples at the various *ex vivo* ages. For each time point and for each blood drawn, RNA from three separate 10 μl bloodstains was isolated. For each subject, therefore, a total of 9 RNA samples were isolated for each time point. Similar-sized samples of dried bloodstains were cut from the fabric, and directly added to 1 ml of Trizol, which was then vortexed briefly and incubated at room temperature for 10 min. Two-hundred microlitres of chloroform was added to the Trizol solution, vortexed for 15 s followed by a room temperature incubation for 3 min. Samples were then centrifuged for 15 min. All centrifugations were performed at 11,000 g at 4 °C. The upper aqueous layer (approx. 500 μl containing the RNA) was transferred to a new tube, and 500 μl of isopropanol was added. The samples were inverted twice, and incubated at room temperature for 10 min. The liquid supernatant was discarded, and 1 ml of 75% ethanol was added to wash the RNA. The samples were briefly vortexed and centrifuged for 5 min. The liquid supernatant was removed and the RNA pellets were allowed to air-dry for 5 min at room temperature. To resuspend the RNA pellet, 40 μl of RNase-free water was added. A sham RNA isolation of cotton containing no bloodstain was preformed with every assay as a negative control.

2.3. Reverse transcription

TaqMan Gold RT-PCR kit (Applied Biosystems, Foster City, CA) was used for the reverse transcription reaction. A reverse transcription master mix was produced (final concentration: 1× TaqMan buffer A; 5.5 mM magnesium chloride; 500 μM each dATP, dCTP, dGTP, and dUTP; 2.5 μM random hexamers), aliquoted into individual PCR tubes, and stored at −20 °C until time of use. Forty microlitres of the RNA suspension, 2.0 μl of RNase inhibitor (0.8 U), and 2.5 μl of multiscribe reverse transcriptase (3.25 U) were added to each reaction. Samples were pulse-centrifuged and placed in a thermocycler Biometro T gradient (Göttingen, Germany) under the following conditions (25 °C for 10 min, 42 °C for 45 min, and 95 °C for 5 min). A no-enzyme control was run with every assay.

2.4. Real-time PCR

A real-time PCR master mix comprising β-actin control reagents (Applied Biosystems), 18S rRNA control reagents (Applied Biosystems), and TaqMan Universal PCR Master Mix was generated to be used for RNA detection. The following list represents the final concentrations of each component of the reaction: 50 nM 18S rRNA forward primer, reverse primer, and 200 nM probe (VIC dye layer); 300 nM β-actin forward primer, reverse primer, and 200 nM probe (FAM dye layer); and 1× TaqMan Universal PCR Master Mix. Real-time PCR master mix (34.75 μl), was added to real-time optical tubes (Applied Biosystems). Each cDNA sample (15.25 μl) was added to the tubes for a total volume of 50 μl. The samples were briefly centrifuged before real-time analysis. Duplicate samples were run for each RNA sample. Samples were placed in a 7300 Sequence Detection System (Applied Biosystems) and run on default conditions. Positive (control cDNA) and negative (water) controls for real-time analysis were included. The sham RNA isolations (cotton cloth lacking a bloodstain) were analyzed at this point for detection of false positives. Analysis of the data was performed using Sequence Detection Software Version 1.4 (Applied Biosystems). Results were analyzed and threshold values adjusted as described by the manufacturer. Control cDNA was used to adjust for experimental variation caused by 7300 machines. All runs were normalized to the same control cDNA before statistical analysis.

Results were exported into Microsoft Excel to generate the values needed for further analysis.

2.5. Statistical analysis

Although samples from the same donor were deposited at different time periods, the results cannot be considered to be totally independent of each other. These samples form a repeated-measures data type, so in common with Anderson et al., we used a mixed linear model for analysis. Factors considered were: gender; donor, nested within gender: (donor [gender]); sample, nested within donor and gender: (sample [donor [gender]]); duplicate number, nested within sample: (duplicate [sample [donor [gender]]]); and age of blood, crossed with gender: (age * gender). All factors except age of blood and gender were taken to be random. Significance tests were based on restricted maximum likelihood methods using Kenward–Roger degree-of-freedom adjustments. To analyze the origin of the variations accurately, we used nested ANOVA (analysis of variance) to determine the variability between donor and within donor. All statistical analysis was carried out using SAS 9.0 software (Cary, North Carolina).

3. Results and discussion

3.1. Stability of 18S and β-actin

In RT-qPCR, the cycle threshold (Ct) value is used to identify the point at which the fluorescent signal exceeds the threshold of detection; i.e., exceeds the background to a statistically significant extent. Over the 28-day period, while the Ct value of 18S did not appreciably change, the Ct value β-actin continued to increase, indicating that it was degrading as a function of time; consequently, the ratio of 18S to β-actin increased over time. Our results supported the hypothesis that there were differences in *ex vivo* RNA decays; specifically, 18S rRNA is stable and actin mRNA decays more rapidly (Fig. 1). Thus, the two-gene marker system in combination has potential for estimating the age of bloodstains.

Anderson et al. (2005) showed a linear relationship between the two genetic markers over 150 days with degradation of 18S beginning after 150 days. Hampson et al. found changes in 18S after 45 days.[25] The differences in stability may be because Anderson et al. kept their samples in a temperature- (25 °C) and humidity- (50%) controlled chamber, while Hampson et al. did not control conditions. We also used uncontrolled conditions, leaving samples at room temperature and room humidity, and with daylight. Our results showed that 18S remained constant over 28 days, while β-actin degraded. This is valuable for forensic practice when the crime happened indoors, but not outdoors.

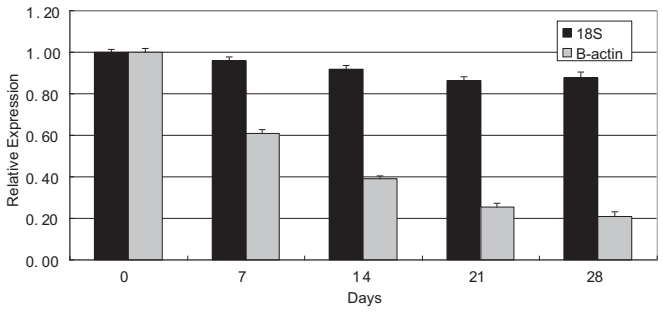

Fig. 1. β-actin degrades in a consistent manner over 28 days, whereas the expression of the 18S species does not change significantly from day 0 to day 28.

3.2. Linear relationship between ratio and bloodstain age

We chose 0, 7, 14, 21, and 28 days as the bloodstain age points: i.e., on weekly intervals. We found a linear relationship between stain age and the ratio (18S:β-actin) (Fig. 2, Table 1). The relationship between the ratio in pooled male and female data and bloodstain age can be estimated as a linear model, thus

$$\text{Mean ratio} = (0.005^{*}\text{Days}) + 1.48.$$

The correlation coefficient of the linear function was high (adjusted $R^2 = 0.981$), which has high statistical significance (F-test: $P = 0.001$). Anderson et al. (2011) chose 0, 6, 30, 90, and 120 days as their study points. Hampson et al. chose 0, 1, 5, 15, 30, 45, 60, 75, and 90 days. It is therefore possible that the technique may be effective over quite long periods of time, as well as for short aging periods under room temperature conditions.

3.3. Variances

The most significant factor contributing to the differences in the results was the age of the bloodstain. The analysis of variation for the age of the bloodstain produced values of 0.83, for the ratio (18S:β-actin). Anderson et al. (2011) found an ANOVA value of 0.93 for the ratio (18S:β-actin), but this may be explained by their temperature- and humidity-controlled experiment .We found that inter-donor variations also showed a linear relationship with age, and the variation seemed to increase with time, with a mean variation between males and females of day 0: 0.0524, and day 28: 0.0733. Similar to our findings, Hampson et al. showed variation increased with time, but Anderson et al. (2005) found variation decreased with time. It was indicated that the most likely cause was the sample storage conditions: Our study and Hampson et al. used uncontrolled room conditions, while Anderson et al. used tightly controlled sample storage and only tested degradation at monthly intervals.

3.4. Gender difference

In this study, the storage conditions for both male and female samples were identical, all samples were blood spots, and the ethnicity of all subjects was similar. Therefore, any differences in stability were due to gender and not ethnic, storage, or sample type differences.

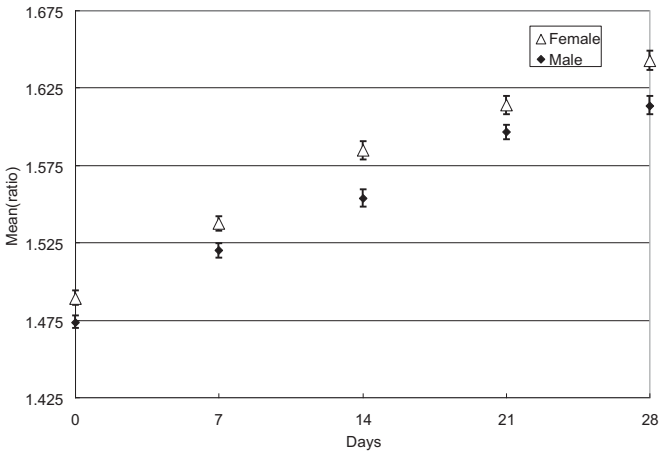

Fig. 2. Analysis of mean (ratio) by age of bloodstain showing the change in RNA levels as a function of bloodstain aging. Data represent the ratio of 18S rRNA to β-actin mRNA as determined by RT-qPCR.

Table 1
Bloodstain 18S rRNA to β-actin mRNA ratios by gender at weekly intervals. The number of subjects in each date set was 8, but as each subject had multiple blood draws, bloodstains per draw, and assays per stain, there were 144 individual samples at each time point.

Sex	Days	*n*	Means of ratio	Standard deviation
F	0	8 (144)	1.489362	0.0595
F	7	8 (144)	1.537252	0.0525
F	14	8 (144)	1.584571	0.0691
F	21	8 (144)	1.613754	0.0716
F	28	8 (144)	1.642756	0.0762
M	0	8 (144)	1.473687	0.0473
M	7	8 (144)	1.519862	0.0586
M	14	8 (144)	1.553657	0.0645
M	21	8 (144)	1.596497	0.0566
M	28	8 (144)	1.613712	0.0704

n = (3 dates of blood draws) (3 bloodstains per date) (2 assays per stain) (8 people) = 144.

Several parameters may affect RNA stability. As it is known that the integrity of RNA may differ between tissue types and between donors. RNA degradation in human brain tissue seems to be accelerated if the deceased received *ante mortem* intensive care and/or the brain was acidotic. This research also identified that regardless of which gene is measured, females, on average, had lower quantities of mRNA in their brain tissue.[24] In bloodstains we found that although female samples had a higher mean ratio, they actually had lower mRNA content than male samples.

Due to the time-dependent changes in ratio, to analyze the gender differences, it was necessary to analyze the mean ratios by individual at each time point (Table 2), and aggregated for male and female subjects (Table 1). These are graphically presented in Fig. 3. Our general mixed linear model indicates that the gender differences are statistically significant. Hampson et al. did not find gender bias-specific effects, but this may be due to tissue origin differences as they used follicle tags, i.e., a group of similar cells, while blood have several different kinds of cells. Anderson et al. (2005) suggested a possible gender-associated difference, but their sample size was too small (4 females and 4 males) for statistical evaluation. Anderson et al. (2011) used samples from 15 males and 15 females over 120 days but found no significant differences between men and women, possibly because of the longer time period, or possibly because of the storage conditions. Finally, different ethnic origins of the donors may have had an effect.

Table 2
Mean ratio of each subject (N = 16) at every time point (0–28 days).

Donor	*n*	0 days	7 days	14 days	21 days	28 days
F1	18	1.5073	1.5432	1.5741	1.5799	1.6361
F2	18	1.4869	1.5210	1.5728	1.5902	1.6601
F3	18	1.4821	1.5493	1.6209	1.6244	1.6219
F4	18	1.4969	1.5459	1.5511	1.6399	1.6657
F5	18	1.4732	1.5327	1.5727	1.6221	1.6879
F6	18	1.4972	1.5332	1.5947	1.6295	1.6260
F7	18	1.5054	1.5218	1.5906	1.6062	1.6309
F8	18	1.4963	1.5509	1.5996	1.6179	1.6134
M1	18	1.4867	1.5315	1.5541	1.5948	1.6315
M2	18	1.4722	1.5451	1.5627	1.5705	1.6242
M3	18	1.4771	1.5104	1.5327	1.6037	1.6195
M4	18	1.4719	1.5068	1.5525	1.6046	1.6131
M5	18	1.4731	1.5281	1.5532	1.5944	1.5880
M6	18	1.4629	1.5332	1.5571	1.6225	1.5901
M7	18	1.4718	1.5185	1.5575	1.5990	1.6050
M8	18	1.4739	1.4854	1.5595	1.5826	1.6383

n = (3 dates of blood draws) (3 bloodstains per date) (2 assays per stain) = 18.

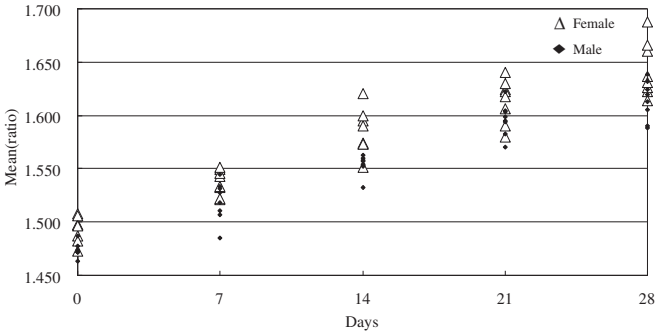

Fig. 3. Analysis of mean (ratio) by age of bloodstain, which shows the change in RNA levels as a function of bloodstain aging. This also shows the difference between males and females.

Whatever the reason for the differences in the data, further research into gender differences needs to be carried out when validating RNA-based methods for analyzing bloodstain age.

3.5. What can be improved

Our results are consistent with those previously reported. We used β-actin as a representative mRNA, but other housekeeping genes such as GAPDH may be equally effective. More research is needed to determine whether RNA related to other gene markers could improve bloodstain age estimation, either individually or in concert by triangulation of several ratio variations.

Another important issue is sample stability: once a bloodstain has been collected from a crime scene, further RNA degradation can be reduced by correct sample storage.

When measuring characteristics such as RNA concentrations, small variations may be the effect of chance and not true differences in gene expression.[26] There may also be differences in gene expression between individuals. This means that any difference in ratio can only be a guide to approximate the age of a bloodstain, not to pinpoint the exact time of deposition. Furthermore, there may be differences in gene expression between individuals which may reduce the utility of the test. Further research should therefore be carried out to identify those RNA species which have minimal variability between individuals. It is also important to explore the environmental effects further (indoors/outdoors, etc.) and the effects of different surfaces (wool, silk, soil, wood, etc.). It is also possible that ethnicity may have effects. We do not believe that this is likely for the RNA species studied here, but until proven otherwise, we believe these results can only be considered to apply to the Chinese Han population.

Ethical approval
None.

Funding
None.

Conflict of interest
None declared.

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